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Characterization of Virulent and Avirulent A/Chicken/Pennsylvania/83 Influenza A Viruses: Potential Role of Defective Interfering RNAs in Nature

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In April 1983, an influenza virus of low virulence appeared in chickens in Pennsylvania. Subsequently, in October 1983, the virus became virulent and caused high mortality in poultry. The causative agent has been identified as an influenza virus of the H5N2 serotype. The hemagglutinin is antigenically closely related to tern/South Africa/61 (H5N3) and the neuraminidase is similar to that from human H2N2 strains (e.g., A/Japan/305/57) and from some avian influenza virus strains (e.g., A/turkey/Mass/66 [H6N2]). Comparison of the genome RNAs of chicken/Penn with other influenza virus isolates by RNA-RNA hybridization indicated that all of the genes of this virus were closely related to those of various other influenza virus isolates from wild birds. Chickens infected with the virulent strain shed high concentrations of virus in their feces (10^7 50% egg infective dose per g), and the virus was isolated from the albumin and yolk of eggs layed just before death. Virus was also isolated from house flies in chicken houses. Serological and virological studies showed that humans are not susceptible to infection with the virus, but can serve as short-term mechanical carriers. Analysis of the RNA of the viruses isolated in April and October by gel migration and RNA-RNA hybridization suggested that these strains were very closely related. Oligonucleotide mapping of the individual genes of virulent and avirulent strains showed a limited number of changes in the genome RNAs, but no consistent differences between the virulent and avirulent strains that could be correlated with pathogenicity were found. Polyacrylamide gel analysis of the early (avirulent) isolates demonstrated the presence of low-molecular-weight RNA bands which is indicative of defective-interfering particles. These RNAs were not present in the virulent isolates. Experimental infection of chickens with mixtures of the avirulent and virulent strains demonstrated that the avirulent virus interferes with the pathogenicity of the virulent virus. The results suggest that the original avirulent virus was probably derived from influenza viruses from wild birds and that the virulent strain was derived from the avirulent strain by selective adaptation rather than by recombination or the introduction of a new virus into the population. This adaptation may have involved the loss of defective RNAs, as well as mutations, and thus provides a possible model for a role of defective-interfering particles in nature.

Influenza virus infections of chickens occur relatively infrequently in the United States; outbreaks of disease associated with high mortality occurred in 1924–1925 and again in 1929 (4) and were caused by a fowl plague-like virus. The last highly pathogenic virus outbreak in domestic poultry in North America occurred in turkeys and was caused by A/turkey/Ontario/6632/66 (H5N9). This virus was so pathogenic that the outbreak of disease was self-limiting (15). Other outbreaks of disease, with clinical signs of mild respiratory infection and low mortality, have occurred infrequently in chickens. A self-limiting outbreak of disease in chickens in Alabama in 1975 was associated with a H4N8 influenza virus (13).

In April 1983, an H5N2 influenza A virus appeared in chickens in Pennsylvania and caused low mortality. Subsequently, in October 1983, virulent influenza viruses were isolated from chickens. The birds showed clinical signs within 48 to 96 h with a mortality of up to 80% after 5 days. The virus was spread to Virginia, and limited outbreaks of disease occurred in New Jersey and Maryland. From November 1983 through June 1984, the virus resulted in the destruction of over 250 flocks of chickens and turkeys, and limited numbers of guinea fowl and chuckars were also affected. Depopulation of infected poultry by a state, federal, and industry Influenza Task Force has resulted in the destruction of over 15 million birds at a cost of approximately $50 million. The Influenza Task Force has been successful in containing the disease to limited areas in Pennsylvania and Virginia. At this time it appears that they will be successful in eradicating this virus from poultry.

We report the characterization of the influenza A viruses that appeared in chickens in April and October 1983.

MATERIALS AND METHODS

Viruses. The following influenza virus isolates from chickens in Pennsylvania were used in this study. A/chicken/Pennsylvania/1/83 was isolated from the index case in April 1983, caused 2.6% mortality and a 31% drop in egg production, and was designated 83-21525 by National Veterinary Services Laboratory (NVSL), Ames, Iowa. A/chicken/Pennsylvania/3/83, was isolated in May 1983, caused 0.6% mortality, and was designated 83-25929 by NVSL. This isolate was plaque-cloned in Madin-Darby canine kidney cultures (25). A/chicken/Pennsylvania/5/83 was isolated in June 1983, caused 9.3% mortality, and was designated 83-26977 by NVSL. A/chicken/Pennsylvania/1370/83 was isolated in October 1983 from a flock of 60,000 laying chickens and caused 1.7% mortality per day and a 50% drop in egg production. These viruses were studied in a containment laboratory (P3) at St. Jude Children's Research Hospital. They were grown

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in 11-day-old chicken embryos and purified by ultrafiltration followed by equilibrium sedimentation as described previously (3). Except as noted below, all virus preparations for RNA analysis were grown with a 1/100 dilution of the previous virus passage and were two to four passages from the initial isolation culture.

High multiplicity passaging of virus. High multiplicity (Von Magnus) virus was prepared as described by Pons and Hirst (22). Undilute infectious allantoic fluid (1 ml) of A/chicken/Pennsylvania/1370/83 influenza virus was inoculated allantoically into 11-day-old embryonated eggs. After 16 to 20 h, the eggs were chilled and the allantoic fluid was harvested. This was used undilute for the second high multiplicity passage.

Animals. White Leghorn chickens (5 to 6 weeks old) and adult laying hens (>6 months old) were used in these studies. The birds were housed in our P3 containment facility in air-filtered compartments. Specific pathogen-free Pekin ducks (1 to 3 months old) were used. They were infected orally or rectally. Tracheal and cloacal swabs were taken as described previously (11).

Specific antibodies. Antisera specific for the isolated hemagglutinin (HA) and neuraminidase (NA) antigens of the reference strains of influenza A viruses were prepared in goats (28). Postinfection chicken antisera to the isolates from chickens were prepared as described previously (20). Monoclonal antibodies to the N2 neuraminidase were prepared by the method of Kohler and Milstein (14) as described by Webster et al. (27).

Serological tests. Hemagglutinin (HA) titrations and hemagglutination inhibition tests were performed in microtiter plates with receptor-destroying enzyme-treated sera (20). Neuraminidase (NA) titrations and NA inhibition tests were done by the procedure of Aymard-Henry et al. (1).

Iodination and electrophoretic analysis of viral RNA. The preparation and iodination of viral RNA have been described previously (3). Labeled viral RNA was resolved on a 3% polyacrylamide gel containing 7 M urea, 50 mM Tris-Borate (pH 8.3), and 1 mM EDTA (17). Electrophoresis was for 4 h at 1200 V. After electrophoresis, the gel was dried and exposed on Kodak XOMAT-AR film.

Competitive hybridization. Genetic homologies were measured by competitive RNA-RNA reassociation as described previously (2). The RNA gene segments of the A/chicken/Pennsylvania/3/83 isolates were isolated by polyacrylamide gel electrophoresis, labeled with ^{125}I, and annealed to homologous complementary RNA in the presence of unlabeled v-RNA from the homologous virus or from other virus strains. Annealing reactions were run at 15°C below the homologous melting temperature. This modest level of stringency was used to show overall levels of homology rather than small differences in base sequence which are amplified when the reaction is run at higher temperatures (24).

Oligonucleotide mapping. Oligonucleotide mapping of ^{32}P-labeled RNase T1 digests of viral genome RNA and isolated genome segments was done by the method of Pedersen and Haseltine (21) as modified by Lee and Fowlks (16). Unincorporated ATP and small digestion products were removed by passage through a 1-ml column of Bio-Gel P-6DG (Bio-Rad Laboratories) followed by two ethanol precipitations. Isolation of viral RNA segments was carried out as described previously (2).

RESULTS

Experimental infection of chickens and virus shedding. The influenza viruses used in this study were provided by NVSL and were designated as low or high pathogenic strains, based on their ability to kill young chickens. A highly pathogenic virus was defined as one that results in not less than 75% mortality within 8 days in at least eight healthy, susceptible chickens, 4 to 8 weeks old, inoculated by the intramuscular, intravenous, or caudal air sac route with bacteria-free infectious allantoic or cell culture fluids (B. C. Easterday, P. A. Bachmann, R. A. Bankowski, V. S. Hinshaw, G. Lang, J. E. Pearson, and R. Rott, Proceedings of the First International Symposium on Avian Influenza, Beltsville, Md., p. VII, 1981). To further compare the pathogenicity of the April and May 1983 isolates with the October 1983 isolates and to measure virus shedding, groups of chickens were inoculated either in the nasal cleft or intravenously. Adult laying hens, inoculated in their nasal clefts with approximately 10^7 50% egg infective doses (EID_{50}) with the avirulent strains (April and May 1983 isolates), shed large quantities of virus in the trachea for 3 to 7 days (up to 10^6 EID_{50} per swab). Virus was recovered sporadically from the feces, and disease signs were absent or mild. On the other hand, when 10-fold larger doses of these viruses were injected intravenously into adult chickens, 50% of the birds died by day 6 postinfection, and virus was recovered from all organs, including the brain.

Adult birds inoculated in the nasal cleft (10^5 EID_{50}) with the virulent virus isolated in October 1983 caused 100% mortality in six birds by day 4 postinoculation, with massive hemorrhage of all tissues but without external disease signs. Adult birds inoculated with lower doses of virus (10^4 EID_{50}) died within 4 to 7 days with more pronounced clinical signs. Virus was isolated from the blood on day 2 postinoculation and from all tissues tested (brain, kidneys, lungs, intestines). There were signs in the birds of central nervous system involvement, which was indicated by the inability to stand and twisting of the head. The birds usually had swollen heads and wattles, with hemorrhage occurring in the intestinal tract. The birds shed high concentrations of virus in their feces with titers of up to 10^7 EID_{50}/g from day 2 postinfection (Table 1). Laying hens continued to produce eggs until the day of death, and the last eggs laid by some birds, contained high levels of virus in the egg white (5.6 EID_{50}/ml) and yolk (3.6 EID_{50}/ml). The mean infectivity titer was obtained from three eggs. Two infected birds (10^5 EID_{50}) and four contact birds layed 37 eggs before they died, and virus was isolated only from 3 eggs layed on the day the birds died. The virus was transmitted among birds in the same cage and among birds in different cages in the same isolation cubicle.

Young birds (5 weeks old) inoculated in the nasal cleft with 10^4 EID_{50} of the virulent virus isolate from October 1983 took longer to die (5 to 9 days), with hemorrhage occurring in the legs and edema in the hocks. Disease signs were less apparent in young birds, and the level of virus shed in the feces was lower (Table 1). Transmission among birds

TABLE 1. Virus shedding in feces from chickens infected with A/chicken/Pennsylvania/1370/83 influenza virus

Bird[a]	Infectivity titer EID_{50}/g on the following days postinfection:					
	1	2	3	4	5	6
Adult hen	<1.0	6.0	7.2	6.5	—[b]	
Young chicken (5 wk)	<1.0	2.5	3.5	3.8	3.8	—

[a] Groups of six chickens each were infected with 10^4 EID_{50}, and pooled fecal samples were collected over a 30-min period each 24 h and titrated in embryonated eggs.

[b] —, Chicken was dead.

TABLE 2. Antigenic characterization of the HA of chicken/Pennsylvania/83 influenza virus

Virus[b]	HA inhibition titers with antisera to the following viruses[a]:					
	Duck/ Alberta/57/75[c]	Tern/South Africa/ 61[d]	Shearwater/ Tyron/ 75[c]	Turkey/ Ontario/ 66[e]	Chicken/ Pennsylvania/ 1/83[c]	Chicken/ Pennsylvania/ 3/83[c]
Duck/Alberta/57/76 (H5N2)	320[f]	640	40	160	160	160
Duck/New York/189/82 (H5N2)	160	320	40	80	80	80
Tn/South Africa/61 (H5N3)	160	640	40	40	80	40
Sh/Tryon/264C/75 (H5N3)	160	640	80	160	80	80
Gull/Maryland/756/78 (H5N9)	80	320	<40	80	40	40
Turkey/Ontario/7732/66 (H5N9)	<40	320	<40	5120	40	<40
Ph/Quebec/643/74 (H5N2)	<40	2560	<40	160	40	<40
Chicken/Scotland/59 (H5N1)	640	1280	80	160	320	320
Chicken/Pennsylvania/1/83 (H5N2)	160	160	<40	40	640	320
Chicken/Pennsylvania/3/83 (H5N2)	320	640	40	80	2560	1280
Chicken/Pennsylvania/1370/83 (H5N2)	160	320	40	40	640	640

[a] HA inhibition titer is the reciprocal of the highest dilution of antiserum inhibiting four hemagglutinating doses of virus.
[b] Viruses inactivated with β-propiolactone.
[c] Chicken antiserum to intact virus.
[d] Goat antiserum to isolated H5 HA.
[e] Rabbit antiserum to intact virus.
[f] Underlined values indicate homologous titers.

in a cage occurred irregularly, and the virus was not transmitted among birds in adjacent cages.

Results of the studies described above show that the influenza virus isolated in April and May 1983 differ markedly in virulence from virus isolated in October 1983 and that laying birds have more pronounced disease signs and shed more virus than do young birds.

Selection of a partially virulent and an avirulent virus from the original A/chicken/Pennslyvania/1/83 isolate. Results of the studies described above indicate that intravenous injection of a large dose of the virus isolated in April 1983 killed half of the chickens that were inoculated, although nasal inoculation of the same virus did not cause any disease signs. It therefore seems possible that the original isolate was a mixture of viruses that differed in virulence. Therefore, studies were done to attempt to isolate a virulent and an avirulent clone from the original isolate. The original virus caused limited mortality in chick embryos (13 of 300 embryos died in 3 days at 35°C). The virus yield from one egg that showed marked hemorrhage of the embryo was passed three times at limiting dilution, each time using embryo death as the endpoint. The virulent virus selected in this way was cloned twice at limiting dilutions in chicken embryos. The avirulent virus was isolated from the original stock by three limiting dilution passages from chick embryos that showed minimal pathology and had an infectivity/HA that was 30-fold lower than that of the virulent virus.

The virulent and avirulent viruses selected as described above were tested for pathogenicity in chick embryos and adult chickens. The virulent clone killed all infected embryos by 5 days postinoculation, whereas the avirulent clone killed only at doses greater than 10^6 EID_{50}. In adult chickens, $10^{7.2}$ EID_{50} of the virulent clone, inoculated into the caudal air sac, killed three of seven birds within 24 h, with extensive hemorrhaging in the ovaries, oviducts, intestines, and lungs. The virus was isolated from the lungs and brains of dead birds. In young birds, the virulent virus was less pathogenic; two of four birds showed clinical signs but none died. The same dose of the avirulent clone caused no disease signs in young or adult birds.

These studies suggest that the original virus is a mixture of viruses with different pathogenicities; some of the clones caused severe clinical disease and mortality, whereas others were nonpathogenic. None of the clones met the criteria described above to define highly pathogenic influenza viruses. The studies also indicate that adult birds are more susceptible than young birds.

Susceptibility of humans and isolation of A/chicken/Pennsylvania/83 influenza virus from flies and pigs. Nasal swabs and serum samples were obtained from persons involved in the depopulation of chickens. H5N2 virus was isolated from 2 of 40 swabs taken immediately after these people left the infected chicken houses but could not be isolated 12 h later (data not shown). Analysis of 109 paired sera showed no increases in antibody. The results indicate that humans are not susceptible to infection with H5N2 influenza virus but can act as short-term vectors. The H5N2 influenza virus was also isolated from a pig that was in close contact with infected chickens. This virus infected pigs experimentally, but there was no evidence for transmission among pigs. The H5N2 virus was isolated from flies caught in the chicken houses of one of the first highly pathogenic virus outbreaks (data not shown).

Antigenic characterization. The HA and NA of the influenza virus isolates were characterized to determine their subtype and whether antigenic variation had occurred between the viruses isolated in May and October 1983. The A/chicken/Pennsylvania/83 viruses were inhibited to high titers by monospecific antisera to the HA of A/tern/South Africa/61 and by other antisera to H5 subtypes (Table 2). Postinfection chicken antisera showed that the HA of the A/chicken/Pennsylvania/83 viruses is also closely related to A/chicken/Scotland/59 (H5N2), A/duck/Alberta/57/76 (H5N2), and A/duck/New York/189/82 (H5N2), but could be distinguished from A/turkey/Ontario/7732/66 (H5N9), A/gull/Maryland/1756/78 (H5N9), and A/shearwater/

TABLE 3. Characterization of NA on A/chicken/Pennsylvania/83 influenza viruses

Virus	NA inhibition titer[a] with the following antibodies:										
	Polyclonal antibodies to:			Monoclonal antibodies to Guiyang/1/57 NA:							
	RI/5+/57[b]	Aichi/2/68[c]	Texas/1/79[c]	4	11	16	19	31	37	39	40
Guiyang/1/57 (H2N2)	560	230	<50	8000	2500	5000	5000	2500	600	1000	800
Texas/1/79 (H3N2)	<50	64	1500	−	−	−	−	−	−	−	−
Turkey/Massachusetts/3740/65 (H6N2)	1000	<50	<50	+[d]	+	+/−	+/−	+	+	+/−	+
Turkey/Minnesota/1574/81 (H5N2)	1800	160	<50	+	+/−	−	+	−	+	−	−
Chicken/Pennsylvania/1/83 (H5N2)	1000	300	<50	+	+	+/−	+/−	+	+	−	+
Chicken/Pennsylvania/3/83 (H5N2)	1000	300	<50	+	+	+/−	+/−	+	+	−	+
Chicken/Pennsylvania/1370/83 (H5N2)	1000	300	<50	+	+	+/−	+/−	+	+	−	+

[a] NA inhibition titer is the reciprocal of the serum dilution causing 50% inhibition of virus giving an optical density of 0.50 at 549 nm.
[b] Goat antiserum to isolated N2 NA.
[c] Rabbit antiserum to intact virus.
[d] NA inhibition titer identical to that obtained with Guiyang/1/57 (H2N2); +/−, NI titer 4-fold less than that obtained with Guiyang/1/57 (H2N2); −, NI titer at least 10-fold or more less than that obtained with Guiyang/1/57 (H2N2).

Tryon/264C/75 (H5N3). The HAs of different nonpathogenic and highly pathogenic isolates of A/chicken/Pennsylvania/83 viruses were indistinguishable when examined with monospecific or postinfection antisera.

The NA on the A/chicken/Pennsylvania/83 viruses was inhibited by monospecific antisera to N2 NA from human influenza isolates obtained from an outbreak in 1957 (Table 3). Antisera to the N2 of more recent human isolates (A/Texas/1/79) failed to inhibit the neuraminidase activity on the A/chicken/Pennsylvania/83 viruses. A panel of monoclonal antibodies to the neuraminidase of A/Guiyang/1/57 (H2N2), a human influenza virus obtained from an outbreak in 1957, were used to characterize the N2 NA on viruses from chickens. The three A/chicken/Pennsylvania/83 influenza viruses and A/Ty/Massachusetts/3240/65 gave similar reactivity patterns with the N2 monoclonal antibodies. Minor antigenic differences were detectable between A/Ty/Minnesota/1574/81 (H5N2) and the A/chicken/Pennsylvania/83 viruses with the N2 monoclonal antibodies; three of the monoclonal antibodies (16, 31, and 40) failed to inhibit the A/Ty/Minnesota/1574/81 virus.

Results of the studies described above indicate that antigenic variation was not detectable between the H5N2 influenza viruses isolated from chickens in April and October 1983. The HA on the A/chicken/Pennsylvania/83 viruses is antigenically similar to that found on the highly virulent A/tern/South Africa/61 and A/chicken/Scotland/59 viruses and on avirulent viruses, such as A/duck/New York/189/82. NA on A/chicken/Pennsylvania/83 viruses was related to the prototype 1957 human N2 NA and typical of the N2 on avian influenza viruses, such as A/turkey/Massachusetts/3740/65 (H5N2).

Analysis of virus RNAs from A/chicken/Pennsylvania/83 influenza viruses. Studies by Sriram et al. (24) have shown that avian influenza viruses possessing antigenically indistinguishable HA and NA glycoproteins can differ significantly in the migration of their RNA segments. The RNA segments from the virulent A/chicken/Pennsylvania/1370/83 virus were indistinguishable from the electrophoretic mobilities of the RNAs from the avirulent A/chicken/Pennsylvania/1/83 virus (Fig. 1). However, the A/chicken/Pennsylvania/1/83 virus showed additional low-molecular-weight RNAs not found in the A/chicken/Pennsylvania/1370/83 virus. The additional low-molecular-weight RNA segments found in A/chicken/Pennsylvania/1/83 virus may be defective-interfering (DI) RNA species, as described by Davis et al. (6). The similar migration patterns of the virion RNAs in the avirulent and virulent chicken/Pennsylvania/83 influenza viruses suggests that the virulent strain was probably not derived by reassortment with other influenza A viruses.

To confirm this, oligonucleotide mapping analyses were done on two avirulent isolates (A/chicken/Pennsylvania/1/83 and A/chicken/Pennsylvania/3/83) and on the virulent isolate (A/chicken/Pennsylvania/1370/83). These results (Fig. 2) indicate that, although some differences can be seen, the three strains compared were very similar, and the two avirulent strains showed about as many oligonucleotide differences among themselves as were seen between the virulent strain and either of the avirulent viruses. To determine the distribution of these changes among the gene segments, RNA segments from each strain were isolated and mapped individually, with the exception of the three P-protein RNAs, which were isolated as a group. The largest DI RNA from chicken/Pennsylvania/3/83 (avirulent) was also nucleotide mapped (Fig. 2G). The results of these analyses are summarized in Table 4. As with the whole RNA maps, the differences between the oligonucleotide maps of the isolated RNAs of the avirulent strains were as great as the differences between the maps of the isolated RNAs of the virulent and avirulent strains. Thus, no correlations could be made between nucleotide differences and virulence. The relative number of nucleotide changes appears to be greater among RNAs 7 and 8; however, the number of changes is too small to allow definitive conclusions to be made. Most of the oligonucleotides of the DI RNA comigrated with those of the P RNAs (Fig. 2). This is consistent with the findings of Davis et al. (6), who showed that defective interfering RNAs were formed by internal deletion of one of the large influenza viral RNAs. The results show that the virulent and avirulent strains are very closely related and suggest that the virulent form of the virus was derived from the avirulent form without the introduction of genes from a new virus.

Interference in vivo. Because the experiments described above suggest the presence of DI particles in the avirulent virus, studies were done to determine whether the avirulent A/chicken/Pennsylvania/1/83 (H5N2) influenza virus could

modify the severity of disease or mortality caused by the highly virulent A/chicken/Pennsylvania/1370/83 (H5N2) influenza virus. Chickens were infected with mixtures of virus containing 10^7 EID_{50} of avirulent virus and 10^4 EID_{50} of virulent virus (Table 5). The virulent virus caused 100% mortality, but when it was mixed with avirulent virus, there was a reduction in severity of disease signs and in mortality; 12 of 14 birds infected with the mixture of viruses survived. On the other hand, if virulent chicken/Pennsylvania/1370/83 influenza virus was mixed with another H5N2 strain, an avirulent strain isolated from wild ducks, A/Michigan/25/80, the majority (five of six) of the animals died. Analysis of the RNA of the duck isolate on polyacrylamide gels indicated that low-molecular-weight RNAs were not detectable (Fig. 1).

These studies indicate that high doses of avirulent chicken/Pennsylvania/1/83 influenza virus that contain low-molecular-weight RNAs can significantly reduce the mortality caused by the virulent duck/Pennsylvania/1370/83 virus, whereas an avirulent virus that did not contain these RNA species did not cause a significant reduction in mortality. Results of the studies suggest that defective interfering particles can interfere with the highly virulent virus and cause reduced mortality.

To further clarify the effect of the small RNAs, the virulent virus was serially passaged in chicken embryos without dilution 15 times and tested for virulence (Table 6). All infected birds showed clinical signs of infection; however, mortality was decreased with the serially passaged virus. The virus from passage 15 was grown and its RNA was analyzed. No discernable small RNAs could be seen (Fig. 1). The virulent variant of the early (low pathogenicity) strain selected in chick embryos, using embryo death as the endpoint, had fewer small RNAs than the avirulent variant generated from the same virus stock by limiting dilution.

Genetic origin. To determine the origin of these viruses, a panel of influenza strains was compared with one of the early isolates, chicken/Pennsylvania/3/83, by competitive RNA-RNA hybridization by using the RNA segments that code for the nonsurface proteins. The panel consisted of 20 avian influenza viruses, including three recent avian strains of the H5N2 serotype (Dk/Minnesota/1545/81, mallard/New York/189/82, and Ty/Minnesota/1180/80) and two equine and one human virus strain (Fig. 3). With each RNA segment, the strains that showed the closest homology were from either wild or domestic birds from North America. One of the H5N2 strains, Ty/Minnesota/1180/80, showed closest homology with segments 5 and 7, but its segment 8 showed no competition with that of chicken/Penn. The virus strain with the most closely related P genes (RNA segments 1, 2, and 3 isolated as a group) was gull/Maryland/5/77. The mammalian strains competed poorly with the chicken/Pennsylvania RNAs with the exception of segment 7, and to a lesser extent, segment 8 of equine/Miami.

Experimental infection of ducks. Because H5N2 viruses have been isolated from asymptomatic infections in wild ducks (10, 11) and some of the gene segments of the A/chicken/Pennsylvania influenza viruses were genetically closely related to influenza viruses from ducks, studies were done to determine whether the chicken/Pennsylvania/83 viruses would replicate in and cause disease in ducks. The avirulent (chicken/Pennsylvania/1/83) and the virulent virus (chicken/Pennsylvania/1370/83) were inoculated into ducks by the oral-tracheal or rectal routes. After oral-tracheal inoculation, virus was isolated from the trachea of some of the ducks for only 1 day postinfection (Table 7), and virus

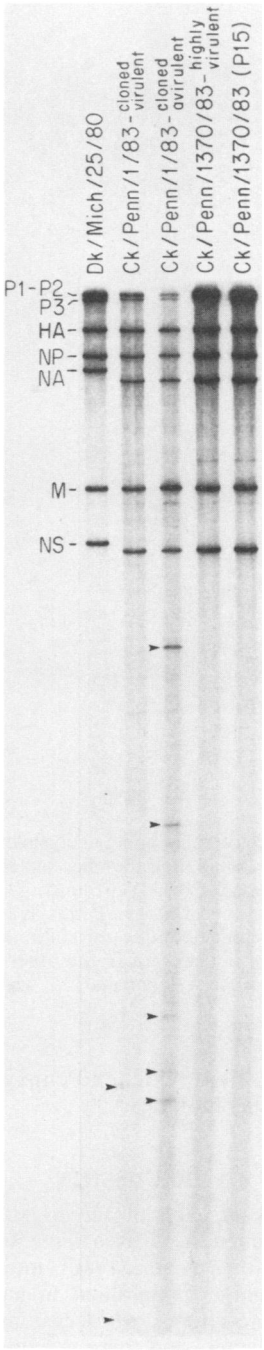

FIG. 1. Polyacrylamide gel analysis of virion RNAs of virulent and avirulent H5N2 virus isolates. Virion RNAs were purified, labeled with I^{125}, and separated as described in the text. Lanes: duck/Michigan/25/80, an avirulent strain not causing interference (Table 6); chicken/Pennsylvania/1/83, passaged in chick embryos, selecting for embryo death; chicken/Pennsylvania/1/83, cloned three times at limiting dilution in chick embryos; chicken/Pennsylvania/1370/83, uncloned virulent virus; chicken/Pennsylvania/1370/83, passaged in chick embryos 15 times without dilution.

was isolated from the feces of 1 of 12 birds inoculated with the virulent virus. Virus was not isolated from other organs. The majority of the ducks had a transitory infection, because 90% of the animals seroconverted. After rectal inoculation, virus was recovered only from the bursal tissue. Neither the

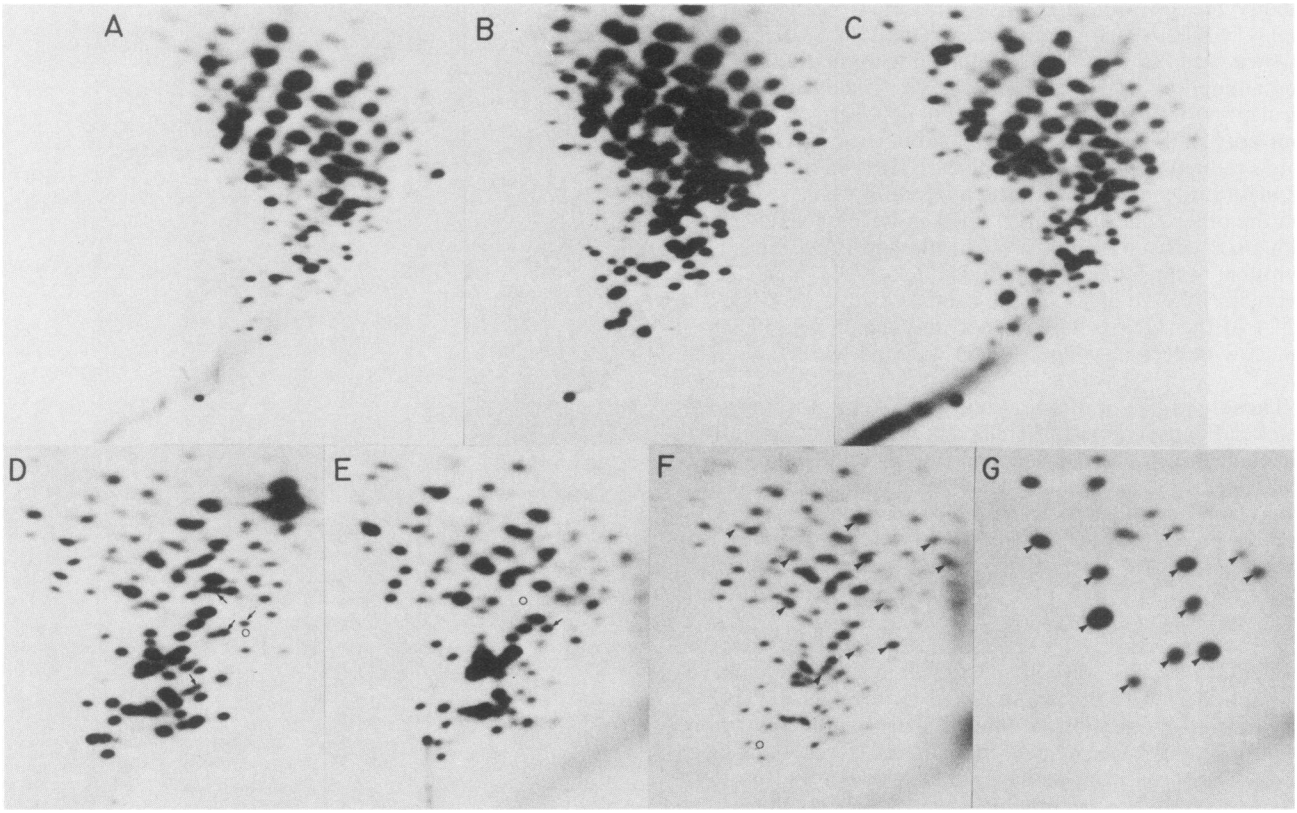

FIG. 2. Oligonucleotide mapping analysis of the RNAs of virulent and avirulent chicken/Pennsylvania isolates. RNAs were digested with RNase T1 and P32 labeled, and the oligonucleotides were separated as described in the text. (A) Chicken/Pennsylvania/1/83 (avirulent). (B) Chicken/Pennsylvania/1370/83 (virulent). (D through F) RNA segments 1, 2, and 3 from chicken/Pennsylvania/1/83, chicken/Pennsylvania/1370/83, and Chicken/Pennsylvania/3/83, respectively. (G) DI RNA isolated from chicken/Pennsylvania/3/83. Arrows in (D), (E), and (F) mark oligonucleotides present in the RNA of one strain but absent in the other two. Circles mark the position of oligonucleotides missing in one strain but present in the other two. Darts in (G) mark oligonucleotides of the DI RNA comigrating with similarly marked oligonucleotides from RNA segments 1, 2, and 3 (F).

avirulent nor virulent chicken/Pennsylvania/83 viruses caused disease signs in ducks.

DISCUSSION

Representatives of each of the 13 subtypes and 9 NA subtypes of influenza A viruses have been isolated from aquatic birds in North America (10), indicating that a large influenza gene pool is maintained in nature. It has been proposed that these viruses are occasionally transmitted to domestic avian species, and there is evidence that this may occur in turkeys (8). The last highly pathogenic avian influenza virus outbreak in North America was A/turkey/Ontario/7732/66 (H5N9). This virus was so pathogenic that the disease was self-limiting (15).

The H5 influenza viruses have been associated with outbreaks of influenza in domestic birds that have resulted in high mortality, i.e., turkey/Ontario/67 and chicken/Scotland/59. The prototype of the H5 subtype was isolated from terns off the coast of South Africa dying of respiratory infection (Tern/South Africa/61). H5 viruses have been isolated from wild ducks regularly, but relatively infrequently when compared with other serotypes. Over the last 8 years, H5 isolates have accounted for only 0.2% of the virus

TABLE 4. Oligonucleotide difference among three isolates of chicken/Pennsylvania

Virus	RNA segment no.[a]:					
	1 to 3	4	5	6	7	8
Chicken/Pennsylvania/1/83 (avirulent)	+4, −1	+1, −1	−1	+1, −1	+1	+2, −3
Chicken/Pennsylvania/3/83 (avirulent)	−1	0	−3	0	−3	−1
Chicken/Pennsylvania/1370/83 (virulent)	+1, −1	0	+1, −1	0	+1, −2	0
Total[b]	72	26	22	16	17	18

[a] Oligonucleotide mapping analysis (as shown with RNAs 1, 2, and 3 in Fig. 1) was done similarly by using the other RNA segments individually. Positive numbers show the number of oligonucleotides present in one strain but absent in the other two. Negative numers show the number of oligonucleotides absent in one strain but present in the other two. A zero indicates that no oligonucleotides were uniquely present or absent.

[b] Total number of oligonucleotides considered in these comparisons.

isolates (V. S. Hinshaw, unpublished data). Their presence in wild birds, however, makes the occasional infection of other species almost inevitable.

The A/chicken/Pennsylvania viruses are closely related antigenically and genetically to viruses recently found in wild and domestic birds. These findings are equivalent to those obtained in the analysis of a seal influenza virus in which it was found that all RNA segments were derived from avian virus isolates, but no strain could be found containing all RNAs that were closely related. The relationship of A/chicken/Pennsylvania RNA 7 and 8 with equine/Miami is also consistent with results obtained with the seal virus (26). Similarly, the lack of homology between RNA 8 of Ty/Minnesota/1180/80 and those of the other avian strains is consistent with the previously described cocirculation of at least two genetically distinct forms of RNA segment 8 in avian influenza viruses (24).

Because H5N2 influenza viruses have been isolated from wild ducks (10, 11), it seems plausible that ducks may have been the original source of the virus. However, because neither the highly pathogenic virus nor the earliest isolate of the avirulent form replicated efficiently in ducks, the virus has apparently undergone sufficient modification to alter its host specificity. The results of the nucleotide mapping indicate that the early isolates, although closely related, differ from each other sufficiently to suggest that the virus may have been in the chicken population for a long enough period of time to accumulate a significant number of mutations. If so, the virus would have had time to adapt to the new host and lose its original host specificity. Presumably, it would have been causing little or no readily apparent disease during this period because no influenza has been noted in chickens in North America for several years. Like the H7N7 seal virus, which also was unable to replicate in the duck intestinal tract, A/chicken/Pennsylvania/83 may be an example of an avian virus strain that was fortuitiously introduced into a new species and underwent a period of adaptation before it became pathogenic and was isolated.

More A/chicken/Pennsylvania/1370/83 influenza virus was shed in the feces of adult birds (up to 10^7 EID_{50}/g) than by juvenile birds (up to 10^5 EID_{50}/g) and may be one of the reasons that approximately twice as many laying hens than broiler chickens were affected. The high concentration of virus in the feces provides huge amounts of virus for transmission within and between flocks. The A/chicken/Pennsylvania/83 (H5N2) virus was isolated from domestic flies caught in the chicken house of one of the earliest highly pathogenic outbreaks. Mechanical transmission of the virus by humans and flies was probably a contributing factor in the spread of this virus.

TABLE 5. Interference between avirulent and virulent A/chicken/Pennsylvania/83 influenza viruses in chickens[a]

H5N2 influenza virus	Dose (EID_{50})	Disease signs (no. affected/no. inoculated)	Mortality (no. dead/no. inoculated)
Chicken/Pennsylvania/1/83 + Chicken/Pennsylvania/1370/83	$10^7 + 10^4$	4/14 (4 to 5)[b]	2/14 (5)
Duck/Michigan/25/80 + Chicken/Pennsylvania/1370/83	$10^7 + 10^4$	6/6 (4)	5/6 (5)
Chicken/Pennsylvania/1370/83	10^4	10/10 (5 to 7)	10/10 (6 to 10)
Duck/Michigan/25/80	10^7	0/2	0/2
Chicken/Pennsylvania/1/83	10^7	0/5	0/5

[a] Groups of White Leghorn chickens (5 weeks old) were infected with influenza viruses by instillation of 0.1 ml into the nasal cleft. The disease signs included sneezing, rales, hemorrhage of the legs and feet, and swelling of the hocks.

[b] Values in parentheses are number of day(s).

TABLE 6. Influence of defective interferring virus on pathogenicity of A/chicken/Pennsylvania/1370/83 influenza virus in chickens

Virus	HA	Infectivity (EID_{50})	Disease signs (no. affected/no. inoculated)	Mortality (no. dead/no. inoculated)
Original	80	7.0	4/4 (5)[b]	4/4 (6 to 10)
P5	15	6.8	4/4 (5)	2/4 (6 to 9)
P10	30	4.8	4/4 (5)	1/4 (6)
P15	8	4.0	4/4 (7)	2/4 (4 to 7)

[a] Groups of four chickens each (5-6 weeks) were inoculated with 0.1 ml of virus in the nasal cleft. The original virus was diluted 10^{-3}, and subsequent passages were inoculated undilute.

[b] Values in parentheses indicate day(s).

Because birds infected with the highly pathogenic strain were viremic within 2 days and virus was isolated from all tissues, it is not surprising that virus should be isolated from eggs laid by infected birds. The presence of infectious virus in the albumen and yolk of eggs layed by hens experimentally infected with Fowl Plague virus has been reported previously (18). The number of infected eggs would be small, but because egg shells are often fed to laying hens, this could provide another mechanism for the transmission of virus to other flocks.

The genetic similarity of the virulent H5N2 strain and the preceding less virulent strains strongly suggest that virulence was acquired by selective adaptation within the population, rather than by the introduction of one or more genes from a different virus. However, we do not know which or how many genes have acquired the mutations that are essential for virulence. Sequence analysis of the HA genes of A/chicken/Pennsylvania/1/83 and A/chicken/Pennsylvania/1370/83 by Kawaoka et al. (13a) has indicated that a change in this gene affecting a potential glycosylation site may be associated with the acquisition of virulence. The roles of the other genes have not been determined. Studies of recombinants between virulent and avirulent strains may determine which genes contain changes that correlate with the acquisition of virulence.

The presence of small RNAs in the avirulent strains of A/chicken/Pennsylvania/83 and the lack of these RNAs in the virulent strain is particularly interesting because this may at least partially explain the sudden emergence of the virulent strain. Although there is a considerable body of evidence about the molecular biology of DI particles of influenza viruses, their role in nature has not been elucidated (19). DI particles have been detected in many virus preparations including vesicular stomatitis virus, Sendai, Simian virus 40, lymphocytic choriomeningitis virus, Rift Valley fever virus, as well as influenza viruses; the DI particles suppress the cytopathic effect of standard virus infection and aid in initiating persistently infected cultures in vitro. Studies with Semliki Forest virus have shown that DI particles protect adult mice from a lethal infection with the parental virus (5, 7). The interference between the low pathogenic and high pathogenic viruses demonstrated in these experiments, together with the reduced mortality of the highly pathogenic strain after high multiplicity passages, supports

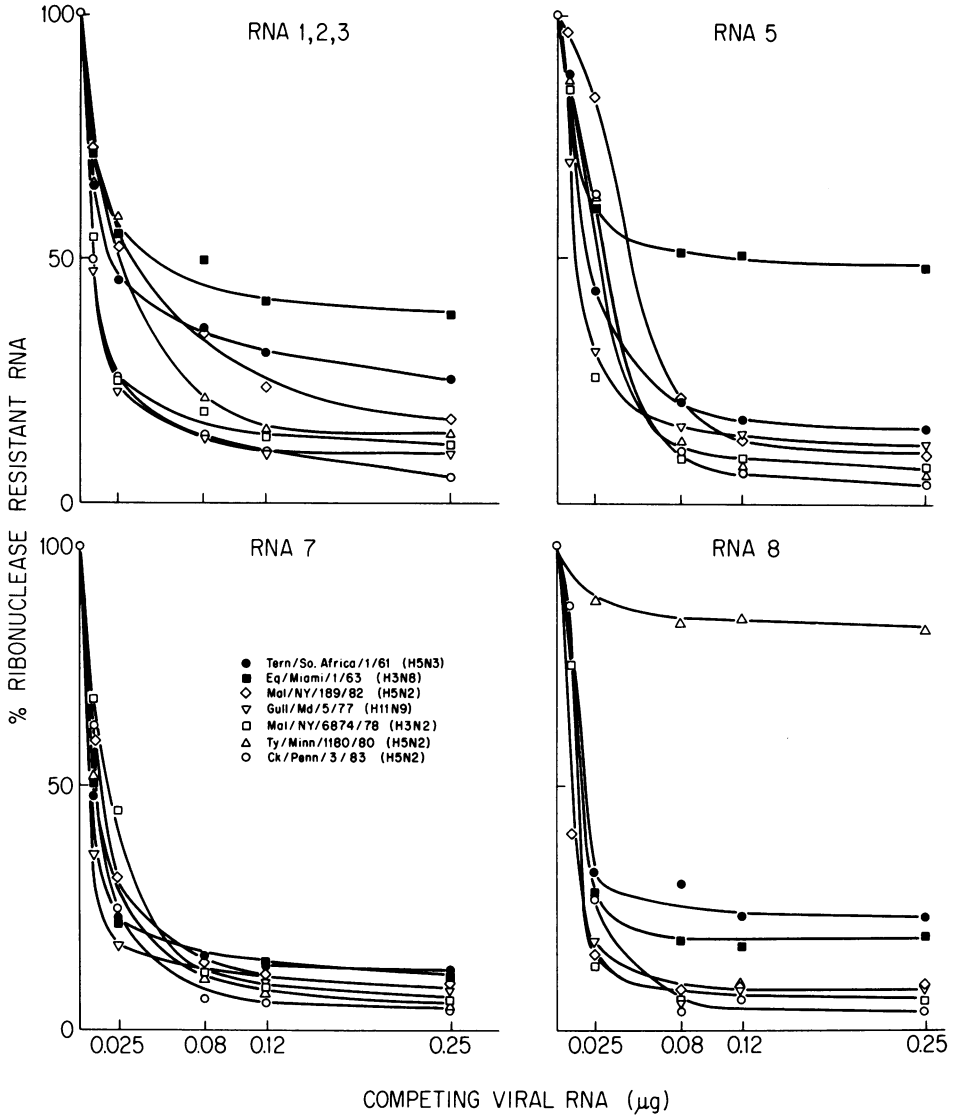

FIG. 3. Competitive hybridization comparison of the RNAs coding for the internal proteins of chicken/Pennsylvania/3/83 with other strains. The genome RNA segments were isolated by polyacrylamide gel electrophoresis, labeled with I^{125}, and allowed to anneal with homologous cRNA in the presence of competing viral RNA from other strains. The efficiency with which the viral RNA competes with the annealing of the reference RNA and its cRNA is a measurement of the relative homology with the reference RNA segment. The results from seven of the strains tested, including those showing closest homology with the RNA segments of chicken/Pennsylvania/3/83, are shown in the figure. The other strains tested, and experimental details, are described in the text.

the contention that defective particles in the original isolate could, in part, be responsible for the reduced mortality caused by this virus in nature.

The presence of distinct DI RNAs was not always a prerequisite for reduced mortality, because high multiplicity passages of virulent virus reduced mortality in experimentally infected chickens without the appearance of small RNAs. However, the ratio of infectivity titer to HA titer was reduced 100-fold, indicating the presence of defective virions. Conversely, selection of virus with increased virulence for chickens by limiting dilution passage did not free the preparation of small RNAs.

Recently, Schubert et al. (23) have shown that a large proportion of full-size vesicular stomatitis virus RNA is defective in that it contains lethal mutations. Like small defective RNAs, the full-size defective RNAs should be propagated in multiply infected cells and also should interfere with the replication of standard viral RNA. They suggest that mutations in the vesicular stomatitis virus polymerase may affect the fidelity of RNA synthesis and thus the number of mutations. The production of full-size defective influenza RNAs could explain the reductions in infectivity and virulence of the virulent A/chicken/Pennsylvania virus after high multiplicity passage. If the ability to produce defective RNAs is a genetically controlled characteristic, as suggested by Schubert et al. (23), then a change in this characteristic may have been a factor in the evolution of the virulent virus.

Since 1979, there have been at least three outbreaks of influenza in animals and birds that resulted in significant mortality. The first occurred in seals in 1979–1980 when up to 20% of these animals died of primary viral pneumonia

TABLE 7. Response of ducks to virulent and avirulent A/chicken/Pennsylvania/83 influenza virus[a]

Virus	Dose (EID_{50})	Route of administration	Virus isolated (no. affected/no. inoculated from the following sites				HA inhibition antibody (no. affected/no. inoculated)
			Trachea	Rectal	Bursal tissue	Disease signs	
Chicken/Pennsylvania/1/83	$10^{3.8}$	Trachea and oral	1/3	0/3	NT[b]	0/3	2/3 (80)[c]
	10^{8}	Trachea and oral	2/2	0/2	0/2	0/2	NA[d]
	10^{8}	Rectal	0/2	0/2	2/2	0/2	NA
Chicken/Pennsylvania/8210/83	$10^{3.8}$	Trachea and oral	1/3	0/3	NT	0/3	2/3 (100)
Chicken/Pennsylvania/1370/83	$10^{7.7}$	Trachea and oral	NT	NT	NT	0/6	
	10^{8}	Trachea and oral	1/12	1/12	NT	1/12	12/12 (1400)
	10^{8}	Rectal	0/1	1/1	1/1	0/1	NA

[a] Pekin white ducks (SPAFAS) (5 months old) were infected with influenza viruses as described in the text.
[b] NT, Not tested.
[c] Values in parentheses give mean HA inhibition antibody titer.
[d] NA, Not available; birds killed for virus analysis on day 3 postinfection.

associated with H7N7 (26). In 1981–1982, and H4N5 influenza virus was associated with up to 15% mortality in seals (9). These viruses, together with the H5N2 virus from chickens in Pennsylvania, appear to have originated from the avian influenza virus gene pool present in wild birds. It also seems likely that the H5N9 virus that caused high mortality in poultry in Ireland (Dennis Alexander, personal communications) also originated from this source. These incidents emphasize the potential importance of this gene pool as a future source of viruses for humans and other species. The transmission of H7N7 influenza viruses to mammals and humans (26) and recent outbreaks of highly pathogenic influenza in domestic poultry suggest that it is only a matter of time before a highly pathogenic virus appears in humans.

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